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Toxicology Research

Influence of the SULT1A status – wild-type, knockout or humanized – on the DNA adduct formation by methyleugenol in extrahepatic tissues of the mouse

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Abstract

Methyleugenol, present in herbs and spices, has demonstrated carcinogenic activity in the liver and, to a lesser extent, in extrahepatic tissues of rats and mice. It forms DNA adducts after hydroxylation and sulphation. As previously reported, hepatic DNA adduct formation by methyleugenol in mice is strongly affected by their sulphotransferase (SULT) 1A status. Now, we analysed the adduct formation in extrahepatic tissues. The time course of the adduct levels was determined in transgenic (tg) mice, expressing human SULT1A1/2, after oral administration of methyleugenol (50 mg/kg body mass). Nearly maximal adduct levels were observed 6 h after treatment. They followed the order: liver > caecum > kidney > colon > stomach > small intestine > lung > spleen. We then selected liver, caecum, kidney and stomach for the main study, in which four mouse lines [wild-type (wt), Sult1a1-knockout (ko), tg, and humanized (ko-tg)] were treated with methyleugenol at varying dose levels. In liver, caecum and kidney, adduct formation was nearly completely dependent on the expression of SULT1A enzymes. In liver, human SULT1A1/2 led to higher adduct levels than mouse Sult1a1, and the effects of both enzymes were approximately additive. In caecum, human SULT1A1/2 and mouse Sult1a1 were nearly equally effective, again with additive effects in tg mice. In kidney, only human SULT1A1/2 played a role: no adducts were detected in wt and ko mice even at the highest dose tested and adduct levels were similar in tg and ko-tg mice. In stomach, adduct formation was unaffected by the SULT1A status. In conclusion: (i) the SULT1A enzymes only affected adduct formation in those tissues in which they are highly expressed (mouse Sult1a1 in liver and caecum, but not in kidney and stomach; human SULT1A1/2 in liver, caecum and kidney, not in stomach of tg mice and humans), indicating a dominating role of local bioactivation; (ii) the additivity of the effects of both enzymes in liver and caecum implies that the enzyme level was limiting in adduct formation; (iii) SULT1A forms dominated the activation of methyleugenol in several tissues, but non-Sult1a1 forms or SULT-independent mechanisms were involved in its adduct formation in stomach.

Abbreviations

CYP, cytochrome P450; dG, 2'-deoxyguanosine; dN, 2'-deoxyribonucleoside(s); ko, *Sult1a1* knockout; ko-tg, *Sult1a1* knockout mice transgenic for human *SULT1A1/2*; LOD, limit of detection; *N*⁶-MIE-dA, *N*⁶-(*trans*-methyloeuogenol-3'-yl)-2'-deoxyadenosine; *N*²-MIE-dG, *N*²-(*trans*-methyloeuogenol-3'-yl)-2'-deoxyguanosine; SULT, sulphotransferase; tg, transgenic for human *SULT1A1/2*; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry; wt, wild-type.

Key words

Alkenylbenzenes; Bioactivation; DNA adducts; Humanized mouse models; SULT1A1

Running head

SULT1A in extrahepatic DNA adduct formation by methyleugenol

Introduction

Methyleugenol, an alkenylbenzene, is a secondary metabolite that has been detected in > 450 plant species from 80 families¹. These data mainly refer to plants used as foods, sources of essential oils or medicines, implying human exposure. Particularly high, although rather variable, levels of methyleugenol are found in many herbs and spices, such as basil, laurel, nutmeg and pimento^{2,3}. Essential oils containing methyleugenol and other alkenylbenzenes are used for flavouring foods and cosmetics⁴. The use of pure methyleugenol for this purpose has been banned in the European Union, but is still legal in the USA. The ban in Europe is owed to the finding that methyleugenol is a genotoxic carcinogen. The liver is the main target organ for the carcinogenicity of various alkenylbenzenes in rat and mouse models⁵⁻⁷. Methyleugenol is most potent regarding hepatocarcinogenicity among the alkenylbenzenes investigated. Moreover, it has demonstrated carcinogenic effects in several additional tissues. Thus, it induced neuroendocrine tumours in the glandular stomach of mice and rats, and renal tubule adenomas, malignant mesotheliomas as well as fibroadenomas in the mammary glands and the skin in rats⁷.

The bioactivation of methyleugenol involves benzylic hydroxylation to 1'-hydroxymethyleugenol by cytochromes P450 (CYPs) and subsequent formation of an electrophilically reactive ester by the action of sulphotransferases (SULTs). *N*²-(*trans*-Methylisoeugenol-3'-yl)-2'-deoxyguanosine (*N*²-MIE-dG) was identified as the major DNA adduct formed by methyleugenol^{8,9}; a second adduct, formed at a nearly 50-fold lower level, was identified as *N*⁶-(*trans*-methylisoeugenol-3'-yl)-2'-deoxyadenosine (*N*⁶-MIE-dA)^{8,9}.

Several human CYP forms are able to hydroxylate methyleugenol in the 1' position, with CYP1A2 showing the highest catalytic efficiency at low substrate concentrations^{10,11}. 1'-Hydroxymethyleugenol constituted a similar percent of the metabolites formed from methyleugenol (100-200 µM) in hepatic microsomal systems from humans, rats, cows and mice (37–51 %) ^{12,13}. Pretreatment of rats with the enzyme inducer Aroclor 1254 strongly enhanced the rate of oxidative metabolism and elevated the contribution of 1' hydroxylation from 37 to 69 %¹². We recently reported that several human SULT forms, expressed in *Salmonella typhimurium* TA100 strains, were able to activate 1'-hydroxymethyleugenol to a mutagen, an effect associated with the

formation of N^2 -MIE-dG and N^6 -MIE-dA adducts in the target cells⁹. Activation was observed with SULT1A1, 1A2, 1E1 and 1C2, but not with the other human SULT forms studied (SULT1A3, 1C1, 1C3, 2A1 and 2B1b). Among the two mouse SULT forms tested, Sult1a1 led to activation, whereas no effect was observed with Sult1d1. In mice *in vivo*, Sult1a1 knockout (ko) drastically reduced the levels of hepatic DNA adducts after treatment with methyleugenol or 1'-hydroxymethyleugenol, compared to the wild-type (wt). Furthermore, transgenic mice for the human *SULT1A1/2* gene cluster, with or without Sult1a1 knockout (ko-tg and tg, respectively), demonstrated clearly higher adduct levels than wt mice¹³.

The number of *SULT* genes in the genomes of humans, mice and rats is similar, approximately 13¹⁴. However, the structure of the *SULT* family markedly differs between these species. For example, four *SULT1A* genes occur in humans, whereas only a single SULT1A gene was detected in the non-primate species investigated, including mice and rats¹⁴. The expression of the individual SULT forms is tissue-dependent¹⁵⁻¹⁸. Liver represents the major source for SULT1A1 in all species investigated, including human, mouse and rat, due to the high expression level¹⁹ and the size of this organ. However, the tissue distribution of orthologous forms can substantially vary between different species. Thus, human *SULT1A1* is expressed many organs, although at varying levels. For example, the level of SULT1A1 protein in human small intestine is similar to the hepatic level. SULT1A1 was also readily detected in human large intestine, lung and kidney^{16, 17, 20}. In contrast, appreciable expression of mouse *Sult1a1*, studied on the mRNA level, was only detected in liver, large intestine and lung among 13 tissues investigated¹⁵. These findings were confirmed on the protein level, with the modification that Sult1a1 protein levels were much higher in liver and large intestine than in lung; the protein was not detected in small intestine, stomach and kidney (W. Meinel and H. R. Glatt, unpublished data).

DNA adducts of methyleugenol are abundant in human liver specimens²¹. DNA adducts of methyleugenol were also detected in human pulmonary DNA²² and colonic DNA (B. H. Monien and H. R. Glatt, unpublished finding).

The observations that methyleugenol is carcinogenic in various extrahepatic tissues of rats and mice and forms DNA adducts in extrahepatic tissues of humans raises the question about the metabolic factors underlying these effects, in particular the role

of SULT1A enzymes. Are they involved in toxicological effects in extrahepatic tissues or are other SULT forms or SULT-independent mechanisms momentous? If SULT1A is important, is it the hepatic enzyme (being the dominating pool in the organism) or the enzyme expressed at the target site?

In order to address these questions, we studied the DNA adduct formation by methyleugenol in extrahepatic tissues (primarily caecum, kidney and stomach) as well as the liver in genetically modified mouse models differing in their SULT1A status. Wild-type (wt) mice express high levels of the endogenous *Sult1a1* protein in liver and caecum, whereas the protein was not detected in stomach and kidney. No *Sult1a1* protein was detected in *Sult1a1*-knockout (ko) mice; this manipulation did not lead to altered expression of other *Sult* forms, studied on the mRNA level in the liver (supplementary material to Ref. ¹⁸). Mice transgenic for the human *SULT1A1/2* gene cluster (tg) express SULT1A1 and SULT1A2 protein in many tissues ¹⁸. Expression of SULT1A1 was generally stronger than that of SULT1A2. The expression level in tg mice followed the order liver > caecum > kidney > stomach (barely detectable expression). All these expression features in the four mentioned tissues of tg mice match the situation observed in humans. The human *SULT1A1/2* transgene did not affect the hepatic levels of *Sult1a1* or other endogenous *Sult* forms (supplementary material to Ref. ¹⁸). We bred ko and tg mice to generated humanised mice for this gene locus, *i.e.* mice containing *SULT1A1/2*, but no functional *Sult1a1* gene (ko-tg).

Materials and methods

Chemicals and enzymes

Methyleugenol, tricapylin, micrococcal nuclease from *Staphylococcus aureus*, phosphodiesterase (type II) from bovine spleen, and alkaline phosphatase from calf intestine were obtained from Sigma-Aldrich (Taufkirchen, Germany). Phosphodiesterase (type II) from calf spleen was purchased from Merck (Darmstadt, Germany). [¹⁵N₅]2'-Deoxyguanosine ([¹⁵N₅]dG) was from Silantes (Munich, Germany). [¹⁵N₅]N²-MIE-dG and [¹⁵N₅]N⁶-MIE-dA were prepared as described previously⁹.

Mouse lines and their maintenance

Wild-type FVB/N mice (subsequently termed wt mice) were purchased from Harlan (Borchen, Germany). The generation of FVB/N mice transgenic for the human *SULT1A1-SULT1A2* gene cluster is described elsewhere¹⁸. The line termed tg1 in the original study was used; it contains multiple copies of human *SULT1A1-SULT1A2* integrated into chromosome 9. The construction of *Sult1a1* knockout mice in the FVB/N background was described in a previous study¹³. The homozygous transgenic line was bred with wt mice to generate animals with a hemizygous gene status with respect to the human transgene (subsequently termed tg mice). The transgenic line tg1 was bred with ko mice to generate a line homozygous for both traits. For the present study, they were bred with ko mice to obtain animals hemizygous for the transgene and homozygous for the knockout (subsequently termed ko-tg mice).

Treatment of animals

All animal experiments presented herein were approved by Landesamt für Umwelt, Gesundheit und Verbraucherschutz of the State of Brandenburg under the number V3-2347-15-2012 or 23-2347-18-2009Ä2. They were maintained under timed lighting conditions (12/12 h). They received water and semisynthetic diet (Altromin C1000 pellets, Altromin, Lage, Germany) *ad libitum*. Male mice of an age of 8–10 weeks were used. Methyleugenol was administered by gavage using tricapylin (1.7 ml/kg body mass) as the vehicle. In the initial experiment, animals were killed at varying times after the treatment with methyleugenol at a dose of 50 mg/kg body mass. Two animals were

used per treatment time in this preliminary experiment. Four additional mice were only treated with the vehicle.

In the main experiment, mice were sacrificed 6 h after the treatment (which is in the plateau phase of the time–response curve) using four different dose levels of methyleugenol (0.05, 0.5, 5 and 50 mg/ kg body mass) plus a group treated only with the vehicle tricapyrin. The number of animals per group was four for each mouse line. Due to shortage of time and resources, four treatment groups (lower two doses in ko and tg animals) could not be analysed. This is irrelevant for the ko mice, as no adducts were detected in any tissue at the next higher dose. The tg mice (expressing both mouse Sult1a1 and SULT1A1/2) were considered to be of lower importance than the ko mice and the strains expressing only SULT1A forms from one species. They were primarily of interest with regard to interactions, which are best studied at the higher doses.

Analysis of DNA adducts

Three methods were used for the isolation of DNA and the sample preparation:

- 1) Initially, nucleic acids were isolated from tissue homogenates using a standard phenol-chloroform extraction procedure²³. The nucleic acids were quantified spectrophotometrically using a NanodropND – 1000 Spectrophotometer (peQlab, Erlangen, Germany). Aliquots of 12.5 µg nucleic acids, spiked with isotope-labelled adduct standards, were digested to nucleosides using phosphodiesterase type II, micrococcal nuclease and alkaline phosphatase, as described previously²⁴.
- 2) When only low adduct levels were expected, sensitivity was enhanced as follows. The amounts of nucleic acids and enzymes used were increased by a factor of nine, and a butanol extraction step was introduced after the digestion in order to enrich the analytes (and internal standards) and to remove disturbing material²¹.
- 3) Method 3 was as method 2, but the DNA was isolated on Qiagen columns (Qiagen, Hilden, Germany), rather than by phenol-chloroform extraction. This approach is more expensive than the other ones, especially if large quantities of DNA are required. It has the advantage that the content of RNA in the isolated nucleic acids is negligible. It was primarily used with tissues containing much RNA, such as liver.

Due to the usage of stable-isotope-labelled internal standards for the adducts and dG (see below), results should be similar with all three methods, apart from differences in the limits of detection (LODs). This was verified with exemplary samples from other studies (see also Ref. ²⁴).

We recently detected that enzymatic digests of DNA prepared by the phenol-chloroform extraction contain substantial levels of ribonucleosides in addition to 2'-deoxyribonucleosides (dN), despite treatment with RNase prior to the extraction ²¹. Fortunately, our analytical method is specific for dN adducts, with no risk of mixing up with ribonucleoside adducts. However, in order to determine the actual level of DNA, we directly determined the level of dG in the hydrolysate. To this end, we spiked small samples of the digests (the equivalent of 24 ng of nucleic acids, omitting the butanol extraction step) with the internal standard [¹⁵N₅]dG (2 pmol) and subjected it to ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis. The total number of dN molecules was calculated knowing that 2'-deoxyguanosine constitutes 21% of all dN in the genomic DNA of the mouse ²⁵.

Adduct and dG levels in the digests were determined with an Acquity UPLC system equipped with an Acquity BEH-Phenyl column (1.7 μm, 2.1 x 100 mm) and connected to a Xevo TQ triple quadrupole mass spectrometer (all from Waters, Eschborn, Germany). Analytes were ionized by an electrospray source operating in the positive ion mode. The *m/z* transitions 428.1 → 177.2 (loss of dA) and 444.1 → 328.2 (loss of deoxyribose) were used for quantifying *N*⁶-MIE-dA and *N*²-MIE-dG, respectively ⁹. Additional *m/z* transitions were used as qualifiers ⁹. Likewise the *m/z* transition 268.2 → 152.1 (loss of deoxyribose) was used for quantifying dG ¹³. Qualifier transitions and settings of the mass spectrometer are given in the supplementary material of a previous study ¹³.

Results and discussion

Absence of *N*⁶-MIE-dA and *N*²-MIE-dG in DNA of negative control animals and LOD

This study contained many mice treated only with the vehicle tricapylin, but not with methyleugenol. None of these negative control animals provided any indication for the presence of *N*⁶-MIE-dA and *N*²-MIE-dG adducts in any tissue. However, noise signals matching the quantifier transition were present in some samples, but were not accompanied by appropriate qualifier transitions. These signals were used for

determining the LODs. They amounted to 10 N^6 -MIE-dA and 30 N^2 -MIE-dG adducts per 10^8 dN for method 1. They were lower for methods 2 and 3, involving increased amounts of DNA per sample and a butanol extraction step. These LODs, determined separately for each tissue from the 16 negative control animals, were 0.5–1.0 N^6 -MIE-dA and 4.0–7.5 N^2 -MIE-dG adducts per 10^8 dN. All protocols provided similar results (within 15 %), when the adduct levels were clearly above the LOD.

Relative levels of N^6 -MIE-dA and N^2 -MIE-dG adducts

The level of the N^2 -MIE-dG was substantially higher than that of N^6 -MIE-dA in all tissue samples when both adducts were detectable. When adduct levels were low, detection was often confined to the N^2 -MIE-dG adducts. When both adduct levels were sufficiently high for accurate quantification ($> 5 \times \text{LOD}$), the ratio of N^2 -MIE-dG to N^6 -MIE-dA adducts was in the range of 38–55. Similar ratios (31–65) were previously recorded in SULT-expressing *S. typhimurium* strains treated with 1'-hydroxymethyleugenol or 3'-hydroxymethylisoeugenol⁹ and human liver specimens²¹. Because the N^2 -MIE-dG adducts were much more abundant and, therefore, could be quantified more accurately than the N^6 -MIE-dA adducts, the subsequent statements primarily refer to the N^2 -MIE-dG adducts. However, they are also true for the N^6 -MIE-dA adducts, when these reached an adequate level.

Time course of the levels of the DNA adducts in eight tissues of tg mice exposed to methyleugenol

The aim of this experiment was to identify extrahepatic tissues susceptible to adduct formation and to determine a treatment duration appropriate for comparing adduct levels formed in different mouse lines. Mice were killed at varying times (1.5–24 h) after the oral administration of methyleugenol. Clear adduct formation was observed in 6 out of 7 extrahepatic tissues tested (Table 1), although the adduct levels remained below those in liver at any time of analysis. In spleen, adducts were only detected at the initial two analysis times. Thereby detection was limited to the N^2 -MIE-dG adducts, their values being only slightly above LOD. With this exception, the time courses of the adduct levels were similar in the remaining seven tissues. Adduct levels were lower at the first analysis time (1.5 h) than at any later times (3–24 h). Maximal or nearly

maximal values were observed after 6 h. From 6 to 12 h, the values remained practically constant. In most tissues this was even the case till the last analysis time, 24 h. However, at this last time point, some decline in the adduct levels (~50 %) was observed in small intestine and caecum, tissues with high cell turnover. Overall, adduct levels in the different tissues of tg mice followed the order: liver > caecum > kidney > colon > stomach > small intestine > lung > spleen.

Impact of the SULT status on the formation of DNA adducts in four tissues of mice treated with methyleugenol at a dose of 50 mg/kg

Based on these findings of the preliminary experiment, we selected a treatment time of 6 h for the main experiment, involving the study of the impact of the SULT1A status. Adducts were analysed in four out of the five tissues with the highest adduct levels in the preliminary experiment. Colon, the remaining tissue in this group, was not analysed due to its similarity with caecum regarding Sult1a1 and SULT1A1/2 expression and other physiological parameters. The four selected tissues differ in SULT1A expression: Liver and caecum show high expression of mouse Sult1a1 and human SULT1A1/2 in the appropriate mouse lines. In kidney, human SULT1A1/2 is well expressed, whereas mouse Sult1a1 is not detectable. In stomach, expression of both enzymes is extremely low.

Knockout of Sult1a1 led to a decrease in the level of hepatic N^2 -MIE-dG adducts by 97 % compared to that found in wt mice (Table 2). An equal decrease of 97 % was observed for the N^6 -MIE-dA adducts. On the contrary, the levels of N^2 -MIE-dG adducts were increased to 513 and 612 %, respectively, in tg-ko and tg mice, respectively. The corresponding numbers for the N^6 -MIE-dA adducts were 381 and 402 %. Thus, adduct formation in liver was nearly completely dependent on the expression of SULT1A enzymes. Human SULT1A1/2 was more effective in this tissue than the endogenous Sult1a1. When both enzymes were present, adduct formation was nearly additive.

Adduct levels in the caecum of wt mice were somewhat higher than in the liver at all four doses tested (Table 3 versus Table 2). Caecal N^2 -MIE-dG adduct levels in ko, tg-ko and tg mice treated with 50 mg/kg amounted to < LOD (2.3 %), 230 % and 97 %, respectively, of that found in wt mice (Table 3). The corresponding numbers for the N^6 -MIE-dA adducts amounted to < LOD (2.5 %), 180 % and 85 %. Thus, the impact of the

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SULT1A status was similar to that observed in liver, except that activation was nearly equally efficient with human SULT1A1/2 and endogenous Sult1a1 in caecum, whereas the human enzyme produced 4–5 times more adducts in liver than the mouse enzyme. The additivity of the effects of mouse Sult1a1 and human SULT1A1/2 in liver and caecum implies that the level of enzyme was limiting in the bioactivation, a situation previously also observed in mutagenicity studies in *S. typhimurium* strains expressing varying levels of human SULT1A1⁹.

No DNA adducts were detected in the kidneys of wt and ko mice treated with methyleugenol (Table 4). However, substantial, nearly equal renal adduct levels were observed in ko-tg and tg mice. At a dose of 50 mg/kg, these levels were \geq 32-fold above LOD for the N^2 -MIE-dG adducts. Thus, only human SULT1A1/2, but not mouse Sult1a1, was able to mediate adduct formation in this organ. This specificity may be due to varying tissue-dependent expression of these enzymes. The mouse enzyme shows very low expression in the kidney¹⁵. On the contrary the human enzyme is well expressed in the kidneys of tg mice¹⁸ as well as humans²⁰.

A fourth situation was found in stomach. The level of adducts formed in this organ was completely unaffected by the SULT1A1 status (Table 5). Indeed, Alnouti and Klaassen¹⁵ found no Sult1a1 expression, studied on the mRNA level, in stomach. Likewise, the levels of SULT1A1/2 protein were very low in stomach tissue of tg mice¹⁸ and humans¹⁶. However, several other Sult forms, such as Sult1b1, 1c1, 1c2, 1d1, 2b1, 3a1 and 5a1, are expressed in mouse stomach, at least on the mRNA level¹⁵. Most of these forms are expressed in liver at lower levels than in stomach, or not at all¹⁵. Sult1d1, expressed in *S. typhimurium* TA100, did not activate 1'-hydroxymethyleugenol to a mutagen⁹. It remains to be examined whether any other Sult forms expressed in mouse stomach is able to activate 1'-hydroxymethyleugenol. We favour this hypothesis, although a SULT-independent bioactivation cannot be ruled out definitely. For example, protonation of the hydroxyl group of 1'-hydroxymethyleugenol could create a leaving group, possibly with generation of reactive 1'-chloromethyleugenol. However, such a mechanism would require that sufficient 1'-hydroxymethyleugenol reaches the acidic and chloride-rich lumen of the stomach.

Dose-response relationships for the formation of DNA adducts by methyleugenol in various tissues of mice differing in their SULT1A status

Methyleugenol was administered at four different dose levels, covering a 1000-fold range, to wt and humanized (ko-tg) mice. The highest two doses were also used in the remaining lines, ko and tg. In the liver, adduct formation was approximately linear with the dose in wt mice. In the liver of humanized (ko-tg) mice adduct formation per dose unit was somewhat enhanced at low dose levels, compared to high doses. In the caecum, adduct formation in wt and ko-tg mice was nearly linear, with a slight increase in the adduct/dose ratio at low doses. Sulphation is considered to be a high-affinity, low-capacity reaction²⁶, a feature that may explain such deviations from linearity.

Interestingly, the dose-response relationships were different in kidney (Table 4) and stomach (Table 5), where the effect per dose unit was enhanced at the highest versus the lower doses. In kidney, substantial adduct formation was only observed in tg and ko-tg mice. When the dose was decreased by a factor of 10, from 50 to 5 mg/kg, renal adduct levels were reduced by a larger factor, 17 and 22, respectively (calculated from N^2 -MIE-dG data in Table 4). We suspect that hydroxylation of methyleugenol primarily occurred in the liver, and that only at high doses some 1'-hydroxymethyleugenol escaped from further metabolism in that tissue and reached the kidneys. Similar findings were encountered in the stomach. When the dose was decreased by a factor of 10, from 50 to 5 mg/kg, adduct levels in stomach were declined to < LOD, *i.e.*, over-proportionally by factors of > 15-19 in the various mouse lines (calculated from N^2 -MIE-dG data in Table 5). Again the relative availability of the proximate genotoxicant, 1'-hydroxymethyleugenol, in stomach may be dose-dependent.

Site of bioactivation

In principle, a reactive metabolite may be formed either within the target tissue, or it is generated elsewhere (*e.g.* in the liver) and transferred to the target site via the circulation. The second possibility – major contribution of systemically available reactive metabolite to the adduct formation – can be excluded for methyleugenol, since the tissue distribution of the adducts formed strongly varied between mouse lines differing in SULT1A status. Some other SULT-dependent genotoxicants behaved differently. An example is 1-hydroxymethylpyrene in the rat. In this species, the renal SULT activity

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toward this substrate amounted to < 1 % of the hepatic activity²⁷. Nevertheless, 1-hydroxymethylpyrene formed higher levels of DNA adducts in kidney than in liver^{27, 28}. Moreover, the reactive metabolite, 1-sulphooxymethylpyrene, could be readily detected in blood serum^{27, 28}. Finally, co-administration of probenecid, an inhibitor of transmembrane transporters for organic anions, strongly enhanced adduct formation by 1-hydroxymethylpyrene in liver (due to inhibited export of its sulpho conjugate) and decreased it in kidney (due to inhibited import)²⁸. Thus, the impact of systemic distribution may differ between different reactive metabolites, even when belonging to the same chemical class, benzylic sulphuric acid esters.

Impact of methyleugenol DNA adducts

In the present study methyleugenol formed particularly high levels of DNA adducts in the liver, the major target tissues of its carcinogenicity. Relatively high adducts levels were also observed in stomach, another target tissue of tumorigenesis in the mouse. 1'-Hydroxysafrole, a congener of 1'-hydroxymethyleugenol forms DNA adducts and induces hepatomas in mouse liver. Boberg *et al.*²⁹ demonstrated that co-administration of pentachlorophenol, an inhibitor of Sult1a1 strongly reduced both effects of 1'-hydroxysafrole. Strong reduction of both effects were also observed in brachymorphic mice, which are genetically deficient in the synthesis of 3'-phosphoadenosine-5'-phosphosulphate, the cofactor for SULTs²⁹.

We previously reported that (+)-1'-hydroxymethyleugenol induces mutations and forms N^2 -MIE-dG to N^6 -MIE-dA adducts in a *S. typhimurium* TA100-derived strain expressing human SULT1A1. All three dose-response curves (for mutations and two adducts) were similarly hyperlinear. Two additional isomers, (-)-1'-hydroxymethyleugenol and (*E*)-3'-hydroxymethylisoeugenol, tested at a single dose, were also mutagenic and formed the same adducts in this strain. The ratios between numbers of induced mutations, N^2 -MIE-dG adducts and N^6 -MIE-dA adducts were rather constant, within the measuring accuracy, between the three test compounds and various dose levels of (+)-1'-hydroxymethyleugenol. Thus, it cannot be decided by correlation analysis which adduct induced the mutations. However, it is known that strain TA100 is generally reverted via mutations at G/C pairs³⁰. Therefore, it is probable that N^2 -MIE-dG adducts induced the reversion mutations in the TA100-derived strain.

However, it should be noted that N^2 -MIE-dG adducts as well as N^6 -MIE-dA adducts involve the modification of sites (exocyclic amino group of purine bases) involved in base pairing. Moreover, they appear to be similarly persistent, as illustrated for example by the time courses presented in Table 1. Therefore, we expect that both adducts are mutagenic. N^2 -MIE-dG than N^6 -MIE-dA may be more important due to its much higher abundance in all models investigated.

Conclusions

We studied the impact of the *SULT1A* status on the methyleugenol-induced DNA adduct formation in four tissues of the mouse. In three tissues (liver, caecum and kidney), this adduct formation was nearly completely (by $\geq 97\%$) dependent on the expression of *SULT1A* enzymes (mouse *Sult1a1* or human *SULT1A1/2*) in that tissue. This dominating influence of *SULT1A* was somewhat surprising, as many additional *Sult* forms are expressed in these tissues¹⁵. However, it is in line with the observation that *SULT1A* enzymes are able to activate 1'-hydroxymethyleugenol at lower concentrations than required with other *SULT* forms⁹. Although adduct formation in stomach was relatively high (rank 5 among 8 tissues studied in tg mice), it was independent from the *SULT1A* status. Thus, it was mediated by other *SULT* form or a *SULT*-independent mechanism.

Whether the *SULT1A* gene status of mice had an impact the methyleugenol-induced adduct formation in given tissue clearly depended on the local expression of the gene. Generally, its impact was strong in the tissues with high expression (human *SULT1A1/2* in liver, caecum and kidney; mouse *Sult1a1* in liver, and caecum). In contrast it had no impact in the tissues with missing or low expression (human *SULT1A1/2* in stomach; mouse *Sult1a1* in stomach and kidney). This finding implies that the final activation step occurred within the target tissue.

In the tissues in which both enzymes, human *SULT1A1/2* and mouse *Sult1a1* are expressed (liver and caecum), their impact on the adduct formation was nearly additive, implying that the amount of enzyme was limiting. *SULT1A1* levels and activities show substantial interindividual variation in humans. Thus Riches *et al.*¹⁷ reported a 11.7-fold variation in the level of *SULT1A1* protein in cytosolic preparations of liver specimens from 28 subjects. The best documented genetic factor affecting the hepatic level of *SULT1A1* protein and activity is gene copy number variation³¹. Indeed, the level of

methyleugenol DNA adducts in a set of 121 liver specimens obtained from different donors was associated with high statistical significance ($p < 10^{-5}$) with the *SULT1A1* gene copy number as well as the SULT1A1 mRNA and protein levels (R. Tremmel, W. Meinel, U. Zanger and H. R. Glatt, manuscript in preparation).

Human SULT1A1 shows broader tissue distribution than their rat and mouse orthologues. Thus, humans may have more target tissues for methyleugenol than these rodent species. In a preliminary experiment, we studied DNA adduct formation by methyleugenol in eight tissues of tg mice, expressing human SULT1A1/2. Adduct formation was close to LOD in spleen, but clearly detected in the remaining seven tissues studied. Somewhat surprisingly adduct formation in lungs of tg mice was only moderate. Pulmonary adduct levels just amounted to 1/49 – 1/38 of those found in liver (calculated from N^2 -MIE-dG data at the various time points presented in Table 1), whereas the median values found in 30 human liver specimens (13 N^2 -MIE-dG per 10^8 dN²¹) and 10 human pulmonary specimens (8.2 N^2 -MIE-dG per 10^8 dN²²) only differed by a factor of 1.6. Of course, humans are exposed chronically via foods and airways, whereas a single oral bolus was used in our experimental model. In addition to SULT1A, the local availability of 1'-hydroxymethyleugenol, the presence of competing or detoxifying enzymes (e.g. UDP-glucuronosyltransferases and glutathione transferases) and other factors may affect the DNA adduct formation in a given tissue and differ between humans and animal models. These factor remain to be investigated.

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Table 1. Time course of N^2 -MIE-dG and N^6 -MIE-dA adduct levels in DNA of various tissues of tg mice treated with methyleugenol ^a

| Time after treatment, h | Adducts per 10 ⁸ dN | | | | | | | |
|-------------------------|--------------------------------|-----------|-----------------|------------|-----------|----------|---------|------------|
| | Liver ^b | Stomach | Small intestine | Caecum | Colon | Lung | Spleen | Kidney |
| N^2 -MIE-dG | | | | | | | | |
| 1.5 | 883 ± 101 | 61 ± 19 | 50 ± 18 | 282 ± 117 | 135 ± 13 | < LOD | 75 ± 6 | 93 ± 5 |
| 3 | 3980 ± 450 | 348 ± 25 | 246 ± 24 | 986 ± 243 | 820 ± 19 | 81 ± 4 | 67 ± 16 | 1070 ± 140 |
| 6 | 5120 ± 410 | 476 ± 136 | 216 ± 30 | 2320 ± 300 | 826 ± 13 | 126 ± 53 | < LOD | 1000 ± 230 |
| 12 | 4400 ± 950 | 470 ± 17 | 225 ± 20 | 2000 ± 110 | 825 ± 224 | 115 ± 60 | < LOD | 1190 ± 280 |
| 24 | 4920 ± 360 | 378 ± 30 | 128 ± 15 | 931 ± 84 | 784 ± 70 | 110 ± 14 | < LOD | 1320 ± 210 |
| N^6 -MIE-dA | | | | | | | | |
| 1.5 | 29 ± 1 | < LOD | < LOD | < LOD | < LOD | < LOD | < LOD | < LOD |
| 3 | 81 ± 12 | 16 ± 1 | < LOD | 33 ± 10 | 24 ± 6 | < LOD | < LOD | 32 ± 3 |
| 6 | 115 ± 4 | 20 ± 2 | < LOD | 61 ± 0 | 29 ± 6 | < LOD | < LOD | 31 ± 11 |
| 12 | 87 ± 37 | 25 ± 5 | < LOD | 51 ± 10 | 25 ± 6 | < LOD | < LOD | 22 ± 4 |
| 24 | 93 ± 12 | 15 ± 2 | < LOD | 23 ± 3 | 39 ± 15 | < LOD | < LOD | 36 ± 3 |

^a Animals were killed at varying times after the oral administration of methyleugenol (50 mg/kg body mass). DNA was enzymatically digested to nucleosides, followed by adduct analysis using isotope-dilution liquid chromatography–tandem mass spectrometry (method 1). Values are mean ± half range of two animals per group. They are only given, if both animals showed adduct levels > LOD (30 and 10 adducts per 10⁸ dN for N^2 -MIE-dG and N^6 -MIE-dA, respectively).

^b These data on adduct levels in liver were already published previously. They are reproduced here to facilitate comparison with adduct levels in extrahepatic tissues (remaining columns).

Table 2. Influence of the SULT1A status on the formation of DNA adducts by methyleugenol in the liver of mice ^a

| Methyleugenol, mg/kg | Adducts per 10 ⁸ dN | | | |
|-------------------------|--------------------------------|------------------------|-------------------------|-------------------------|
| | wt | ko | tg | ko-tg |
| | <i>N</i> ² -MIE-dG | | | |
| 0 | < LOD ³ | < LOD ³ | < LOD ³ | < LOD ³ |
| 0.05 | < LOD ³ | – | – | 13 ± 7 ³ |
| 0.5 | 8 ± 2 ³ | – | – | 52 ± 20 ¹ |
| 5 | 100 ± 22 ¹ | < LOD ³ | 417 ± 76 ¹ | 385 ± 25 ¹ |
| 50 | 735 ± 342 ¹ | 23 ± 2 ³ | 4500 ± 180 ¹ | 3770 ± 320 ¹ |
| | <i>N</i> ⁶ -MIE-dA | | | |
| 0 | < LOD ³ | < LOD ³ | < LOD ³ | < LOD ³ |
| 0.05 | < LOD ³ | – | – | 1.3 ± 0.6 ³ |
| 0.5 | < LOD ³ | – | – | < LOD ¹ |
| 5 | < LOD ¹ | < LOD ³ | < LOD ¹ | < LOD ¹ |
| 50 | 21 ± 10 ¹ | 0.6 ± 0.2 ³ | 81 ± 13 ¹ | 84 ± 21 ¹ |

^a These data were already published previously. They are reproduced here to facilitate comparison with adduct levels in extrahepatic tissues (Tables 3-5). Animals were killed 6 h after the oral administration of methyleugenol. Values are mean ± SD of 4 animals. They are only given, if at least 3 out of the 4 animals showed adducts levels > LOD. –, not investigated.

¹ Method 1 (12.5 µg DNA) was used. LOD: 30 *N*²-MIE-dG and 10 *N*⁶-MIE-dA adducts per 10⁸ dN.

³ Method 3 (112.5 µg DNA) was used. LOD (for liver): 6 *N*²-MIE-dG and 0.6 *N*⁶-MIE-dA adducts per 10⁸ dN.

Table 3. Influence of the SULT1A status on the formation of DNA adducts by methyleugenol in the caecum of mice ^a

| Methyleugenol, mg/kg | Adducts per 10 ⁸ dN | | | |
|-------------------------|--------------------------------|--------------------|-------------------------|-------------------------|
| | wt | ko | tg | ko-tg |
| | <i>N</i> ² -MIE-dG | | | |
| 0 | < LOD ² | < LOD ² | < LOD ² | < LOD ² |
| 0.05 | 6 ± 1 ² | – | – | 7 ± 3 ² |
| 0.5 | < LOD ¹ | – | – | < LOD ¹ |
| 5 | 96 ± 14 ¹ | < LOD ¹ | 293 ± 61 ¹ | 100 ± 37 ¹ |
| 50 | 1210 ± 740 ¹ | < LOD ¹ | 2780 ± 330 ¹ | 1170 ± 310 ¹ |
| | <i>N</i> ⁶ -MIE-dA | | | |
| 0 | < LOD ² | < LOD ² | < LOD ² | < LOD ² |
| 0.05 | 1 ± 0 ² | – | – | < LOD ² |
| 0.5 | < LOD ¹ | – | – | < LOD ¹ |
| 5 | < LOD ¹ | < LOD ¹ | < LOD ¹ | < LOD ¹ |
| 50 | 40 ± 22 ¹ | < LOD ¹ | 72 ± 7 ¹ | 34 ± 6 ¹ |

^a Animals were killed 6 h after the oral administration of methyleugenol. Values are mean ± SD of 4 animals. They are only given, if at least 3 out of the 4 animals showed adducts levels > LOD. –, not investigated.

¹ Method 1 (12.5 µg DNA) was used. LOD: 30 *N*²-MIE-dG and 10 *N*⁶-MIE-dA adducts per 10⁸ dN.

² Method 2 (112.5 µg DNA) was used. LOD (for caecum): 4.0 *N*²-MIE-dG and 1.0 *N*⁶-MIE-dA adducts per 10⁸ dN.

Table 4. Influence of the SULT1A status on the formation of DNA adducts by methyleugenol in the kidneys of mice ^a

| Methyleugenol, mg/kg | Adducts per 10 ⁸ dN | | | |
|-------------------------|--------------------------------|--------------------|----------------------|-------------------------|
| | wt | ko | tg | ko-tg |
| | <i>N</i> ² -MIE-dG | | | |
| 0 | < LOD ² | < LOD ² | < LOD ² | < LOD ² |
| 0.05 | < LOD ² | – | – | < LOD ² |
| 0.5 | < LOD ¹ | – | – | < LOD ¹ |
| 5 | < LOD ¹ | < LOD ¹ | 57 ± 17 ¹ | 50 ± 11 ¹ |
| 50 | < LOD ¹ | < LOD ¹ | 950 ± 70 | 1110 ± 320 ¹ |
| | <i>N</i> ⁶ -MIE-dA | | | |
| 0 | < LOD ² | < LOD ² | < LOD ² | < LOD ² |
| 0.05 | < LOD ² | – | – | < LOD ² |
| 0.5 | < LOD ¹ | – | – | < LOD ¹ |
| 5 | < LOD ¹ | < LOD ¹ | < LOD ¹ | < LOD ¹ |
| 50 | < LOD ¹ | < LOD ¹ | 19 ± 5 ¹ | 18 ± 10 ¹ |

^a Animals were killed 6 h after the oral administration of methyleugenol. Values are mean ± SD of 4 animals. They are only given, if at least 3 out of the 4 animals showed adducts levels > LOD. –, not investigated.

¹ Method 1 (12.5 µg DNA) was used. LOD: 30 *N*²-MIE-dG and 10 *N*⁶-MIE-dA adducts per 10⁸ dN.

² Method 2 (112.5 µg DNA) was used. LOD (for kidney): 4.7 *N*²-MIE-dG and 0.5 *N*⁶-MIE-dA adducts per 10⁸ dN.

Table 5. Influence of the SULT1A status on the formation of DNA adducts by methyleugenol in the stomach of mice ^a

| Methyleugenol, mg/kg | Adducts per 10 ⁸ dN | | | |
|-------------------------|--------------------------------|------------------------|------------------------|-----------------------|
| | wt | ko | tg | ko-tg |
| | <i>N</i> ² -MIE-dG | | | |
| 0 | < LOD ² | < LOD ² | < LOD ² | < LOD ² |
| 0.05 | < LOD ² | – | – | < LOD ² |
| 0.5 | < LOD ¹ | – | – | < LOD ¹ |
| 5 | < LOD ¹ | < LOD ¹ | 32 ± 9 ¹ | < LOD ¹ |
| 50 | 520 ± 350 ¹ | 540 ± 190 ¹ | 580 ± 120 ¹ | 550 ± 90 ¹ |
| | <i>N</i> ⁶ -MIE-dA | | | |
| 0 | < LOD ² | < LOD ² | < LOD ² | < LOD ² |
| 0.05 | < LOD ² | – | – | < LOD ² |
| 0.5 | < LOD ¹ | – | – | < LOD ¹ |
| 5 | < LOD ¹ | < LOD ¹ | < LOD ¹ | < LOD ¹ |
| 50 | 19 ± 12 ¹ | 26 ± 14 ¹ | 24 ± 9 ¹ | 24 ± 7 ¹ |

^a Animals were killed 6 h after the oral administration of methyleugenol. Values are mean ± SD of 4 animals. They are only given, if at least 3 out of the 4 animals showed adducts levels > LOD. –, not investigated.

¹ Method 1 (12.5 µg DNA) was used. LOD: 30 *N*²-MIE-dG and 10 *N*⁶-MIE-dA adducts per 10⁸ dN.

² Method 2 (112.5 µg DNA) was used. LOD (for kidney): 7.5 *N*²-MIE-dG and 1.0 *N*⁶-MIE-dA adducts per 10⁸ dN.